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# Rapid Viability Polymerase Chain Reaction Method for Detection of virulent *Bacillus anthracis* from Environmental Samples

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## ABSTRACT

In the event of a biothreat agent release, hundreds of samples would need to be rapidly processed to characterize the extent of contamination and determine the efficacy of remediation activities. Current biological agent identification and viability determination methods are both labor- and time-intensive such that turnaround time for confirmed results is typically several days. In order to alleviate this critical issue, automated, high throughput sample processing methods were developed in which real time Polymerase Chain Reaction (PCR) analysis is conducted on samples before and after incubation. The method, referred to as Rapid Viability (RV)-PCR, uses the change in cycle threshold after incubation to detect the presence of live organisms. In this article, we report a novel RV-PCR method for detection of live, virulent *Bacillus anthracis*, in which the incubation time was drastically reduced, from 14 hr, down to 9 hr, bringing the total turnaround time for results below 15 hr. The method incorporates a magnetic bead-based DNA clean up step prior to PCR analysis, as well as specific real-time PCR assays for the *B. anthracis* chromosome and pXO1 and pXO2 plasmids.

Verification of the method applied to both manual and automated detection of virulent *B. anthracis* was conducted with a total of 192 wipe, air filter and water samples. The verification included challenges with high populations of non-target microorganisms, high populations of dead *B. anthracis* spores and high loadings of debris, and performance criteria included limit of detection, accuracy with plating and turnaround time for results. A detection level of 10 CFU/sample was demonstrated with both manual and automated RV-PCR methods in the presence of all challenges. Experiments exploring the relationship between the incubation time

and the limit of detection suggest that the method could be further shortened by an additional 2-3 hr for relatively clean samples.

## INTRODUCTION

If a biothreat agent were to be released, hundreds to thousands of environmental samples of diverse types would need to be rapidly processed and analyzed in order to first characterize the contamination of the site and then assess the effectiveness of decontamination activities. Decision-makers also need rapid results for re-mobilizing disinfection equipment in the case of incomplete decontamination and for re-opening facilities and areas based on results from clearance sampling (1-3). The need for biological agent identification methods with greater sensitivity, specificity, and speed was stressed in a review of technology challenges associated with responding to biological attacks in the civilian sector (4), along with the need for automated methods to improve sample and analysis throughput while reducing costs and potential personnel exposure to a biological warfare agent.

Current methods used by the Centers for Diseases Control and Prevention (CDC) to assess the viability of spores on surfaces rely on culturing samples on solid media (5, 6). These methods involve several manual steps including pipetting to prepare dilution series, plating of numerous replicates for a series of dilutions, and colony counting, which make it labor-, space- and time-intensive. Typically, only 30-40 samples are processed each day with confirmed results obtained days later (5, 6). Validated, rapid viability test protocols are therefore needed to ensure public safety and to help mitigate impacts due to facility closures following a biothreat agent release. This critical need was highlighted during the response to the 2001 Anthrax attacks in which clearance sampling and analysis required excessive time prior to facilities re-opening (4).

68 Because risk assessment after such an attack is determined on the basis of the presence of viable  
69 spore populations, and because DNA and antigenic materials remain after decontamination (7), it  
70 is critical to determine viability rather than simply the presence of nucleic acid from a pathogen.  
71 We leveraged the useful features of real-time Polymerase Chain Reaction (PCR) and expanded  
72 its capabilities by conducting PCR analysis before and after incubating samples, and using the  
73 change in PCR response to indicate the presence of viable spores or cells. The approach,  
74 referred to as Rapid Viability (RV)-PCR, uses accepted methods including culturing and real-  
75 time PCR analysis (although in a different format) to allow more rapid and specific analysis.  
76 High throughput sample processing is enabled by commercial automation in combination with  
77 96-well real-time PCR analysis, leading to the processing of hundreds of surface samples per day  
78 with results achieved in less than 24 hr.

79 Initial RV-PCR protocols were developed and tested with surrogate organisms including *Bacillus*  
80 *atrophaeus* and the non-virulent *Bacillus anthracis* Sterne strain. In these experiments, detection  
81 of low levels of viable spores (1-10 CFU/sample) was demonstrated for various sample types  
82 (wipes, swabs, and vacuum filters) in the presence of environmental backgrounds, high  
83 populations of live non-target spores/micro-organisms, and dead target spores killed by chlorine  
84 dioxide fumigation (8). Hundreds of samples were processed, demonstrating high throughput  
85 analysis and similar detection limits and accuracy as traditional viability analysis. The Most-  
86 Probable-Number Rapid Viability (MPN-RV) PCR, a method variation in which replicates of  
87 dilution series are analyzed to provide a quantitative estimate of the spore levels, was also tested  
88 alongside the traditional culture method for the quantification of *B. anthracis* Sterne spores in  
89 macrofoam swabs from a multi-center validation study conducted by the CDC (9). MPN RV-  
90 PCR provided correct identification for all samples analyzed in this study in less than 24 hr and

the estimation of the number of spores by MPN RV-PCR was within the order of magnitude of the values determined using the traditional culture method (6).

This manuscript describes optimized automated and manual methods for the detection of virulent *B. anthracis* Ames in wipes, air filters, and water samples. Real-time PCR assays targeting the chromosome and both plasmids were down-selected *in silico*, experimentally optimized, and tested for selectivity, sensitivity, and robustness in the presence of growth medium and cell debris before being integrated in the RV-PCR method. The new method endpoint was shortened from its initial overnight incubation (14 hr) to 9 hr by performing a magnetic bead-based DNA clean up before PCR analysis, bringing the turnaround time for results below 15 hr for 24 samples (each additional set of 24 samples would require an additional 2-3 hr of processing time when proceeding with serial analysis with a single robot and personnel working in shifts) while maintaining the limit of detection at the level of 10 CFU/sample. Testing included challenges with high populations of live non-target micro-organisms, high populations of dead *B. anthracis* spores, and high loadings of debris. Criteria included limit of detection, accuracy with plating, and turn-around time for results. Experiments demonstrated a detection level of 10 CFU/sample with both manual and automated RV-PCR methods in the presence of all challenges. Additional experiments exploring the relationship between the incubation time and the limit of detection suggest that the method could be shortened by another 2-3 hr when analyzing relatively clean samples.

## MATERIALS AND METHODS

**Bacterial strain and culture conditions.** The pathogenic *B. anthracis* Ames strain was cultivated in Brain Heart Infusion (BHI) medium and on BHI agar plates. Spore stocks were stored in a 70% water and 30% ethanol solution at 4 °C.

***B. anthracis* Ames spore preparation.** *B. anthracis* was streaked for growth onto BHI agar and incubated overnight at 36 °C. The organism was then streaked and incubated a second time for isolation. A  $10^8$  CFU/mL suspension of the 24 hr growth was prepared in phosphate buffer (25mM  $\text{KH}_2\text{PO}_4$ , pH 7.2), plated onto soil extract beef peptone agar and incubated at 36 °C until 99% sporulation was achieved. Plates were then scraped and rinsed using sterile water and a cell scraper (the content of each plate was transferred to a 50 mL centrifuge tube in a total of 30 mL of water). The spore preparation was cleaned using vortexing (2 min), centrifugation (4000 rpm for 15 min), removal of the supernatant and addition of sterile water. This cleanup procedure was repeated 4 times. Twenty milliliter of a 1:1 (ethanol:water) solution were then added to the centrifuge tubes, which were vortexed for 2 min to re-suspend the spore pellets. Tubes were then placed on a shaker platform for 1 hr at 80 rpm. After this step, the spore suspension was washed again 7 consecutive times using the vortexing, centrifugation and supernatant exchange technique described above. The suspension titer after these washing steps was  $10^9$  CFU/mL, as measured by plating, and the fraction of dead spores, measured by microscopy was < 1%. The final spore re-suspension was performed using a mixture of 70% water and 30% ethanol in order to generate a spore stock for storage at 4 °C.

**Sample spiking.** Prior to each RV-PCR experiment, the *B. anthracis* working spore stock ( $10^4$  CFU/mL) was vortexed on a platform vortexer (VWR International, Model VX-2500) for 20 min. Successive 10-fold dilutions were prepared in phosphate buffer (25 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4),

down to  $10^2$  CFU/mL. Three replicates each of the  $10^2$  and  $10^3$  CFU/mL were cultured on agar plates following the traditional viability protocol described below. Typically, 100  $\mu$ L of the  $10^2$  CFU/mL dilution and 50  $\mu$ L of the  $10^3$  CFU/mL dilution were plated in triplicate, in order to determine the actual inoculation levels. Water (sterile, deionized water), air filter (Millipore, Cat. No. FSLW04700) and wipe samples (VWR International, Cat. No. 8052) were inoculated using 100  $\mu$ L of the  $10^2$  CFU/mL suspension (10 CFU/sample level) and 100  $\mu$ L of the  $10^3$  CFU/mL suspension (100 CFU/sample level).

The targeted levels for this study were the 10 spore level (1 to 99 CFU/sample) and the 100 spore level (100 to 999 CFU/sample). Although the goal was to test the lowest spore numbers for each level, variability with pipetting, vortexing, and surface binding of the spores to stock tubes generated slightly different CFU values for each experiment, which were quantified by systematic plating of the spiking solutions prior to sample inoculation.

**Preparation of dirty wipes.** The well-characterized Arizona Fine Test Dust (Powder Technology Inc., Burnsville, MN) was used for this study. The material consists of Arizona sand including Arizona Road Dust, Arizona Silica, Air Cleaner Fine and Air Cleaner Coarse Test Dusts, Society of Automotive Engineers Fine and Coarse Test Dusts, J726 Test Dusts, ISO Ultrafine, ISO Fine, ISO Medium and ISO Coarse Test Dusts, and MIL STD 810 Blowing Dust (10). Analysis of chemical composition performed by the manufacturer indicates that the material consists of:  $\text{SiO}_2$  (68 to 76%),  $\text{Al}_2\text{O}_3$  (10 to 15%),  $\text{Fe}_2\text{O}_3$  (2 to 5%),  $\text{Na}_2\text{O}$  (2 to 4%),  $\text{CaO}$  (2 to 5%),  $\text{MgO}$  (1 to 2%),  $\text{TiO}_2$  (0.5 to 1.0%), and  $\text{K}_2\text{O}$  (2 to 5%). Microbial characterization of the test dust performed by the CDC found 39 morphologically distinct colony types including *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus mycoides*, *Bacillus endophyticus*, *actinomycetes*, molds, yeast, *micrococcus* and *streptomyces* (11). A 0.5 mg/mL test dust slurry stock was

prepared by weighing 10 g of test dust in a conical tube, adding 20 mL of deionized water, and vortexing at high speed for 20 min. Five hundred microliters of slurry were added to wipes (250 mg of dust) as a challenge.

**Preparation of dirty air filters.** Air filters collected from portable air sampling units that operated for 24 hr at approx. 200 lpm, were used as challenges for this study. Filters contained debris from both indoor and outdoor environments and were spiked as received.

**Preparation of chemically spiked water samples.** The water used in this study was filtered through a Milli-Q™ water system (Millipore Co., Billerica, MA). Challenge samples were prepared by adding ferrous sulfate and humic acid at levels of 10 mg/L (Sigma-Aldrich Co., Cat. No. 53680 and F8048).

**Addition of dead *B. anthracis* Ames spore background.** A stock of *B. anthracis* Ames spores ( $10^6$  CFU/mL confirmed by plate counts) was killed by autoclaving three times at 126 °C and 15 psi for 30 min. Six 100 µL aliquots were cultured on solid BHI medium and incubated for 48 hr to confirm non-viability of the stock. One milliliter of the dead spore stock solution ( $10^6$  dead spores/sample) was added to each sample type as a challenge.

**Addition of live non-target background.** A stock of *Bacillus atrophaeus* (ATCC No. 9372) was plated to confirm concentration at the level of  $10^4$  CFU/mL. One hundred micro-liters ( $10^3$  CFU/sample) were then inoculated on each sample type as a live challenge. *Pseudomonas aeruginosa* (ATCC No. 10145) cells were grown overnight in a shake flask and diluted to a concentration of  $10^7$  CFU/mL using optical density measurements at 620 nm to assess the cell concentration. One hundred microliter ( $10^6$  CFU/sample) of this diluted culture were inoculated on each sample. The final live background for each sample was a combination of  $10^3$  *B. atrophaeus* spores and  $10^6$  *P. aeruginosa* cells.

**Traditional viability.** For traditional viability analysis, 2 to 3 successive ten-fold dilutions were cultured on BHI agar and incubated overnight at 30 °C. Three 100 µL replicates were plated for each sample. Colony counts were obtained the next day and corrected for dilution.

**Rapid-Viability PCR.** The experimental protocol outline as well as pictures of the equipment used, are provided in Figure 1. Briefly, samples were placed in 30 mL conical tubes and spiked with *B. anthracis* Ames as described above. A mesh support [Mc Master Carr Inc., Cat. No. 93185 T17; 2.75-inch (6.98-cm) diameter, 0.033-inch (~0.084-cm) openings] was used to maintain wipe and air filter samples to the side of the tube, clear of pipetting. Twenty milliliters of extraction buffer (70% of 0.25 mM KH<sub>2</sub>PO<sub>4</sub>/0.1% Tween 80 [pH 7.2] and 30% ethanol; final pH ~9.5) was added to each tube (for wipes and filters) and the tubes were vortexed for 20 min on a platform vortexer to remove spores from the sample matrix. Thirteen milliliters were then transferred from each sample tube to a filter cup (when performing the protocol manually, 15 mL were pipetted out of the sample tube and 13 mL were dispensed into the filter cup, to minimize the probability of aerosol formation with select agents; the same volume was transferred with robotics, to provide consistency). Spores suspended in the extraction buffer were then collected on the 0.45 µm filter of the filter cups using a vacuum manifold and a vacuum pump (0.45 µm filters provided faster filtration in the presence of dirt and debris while generating results that were not statistically different from 0.22 µm filters) (12). Filters were washed with 7 mL of filter-sterilized 210 mM KH<sub>2</sub>PO<sub>4</sub> buffer (pH 6.0) followed by 3 mL of filter-sterilized 25 mM KH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.2). Filter cups were then sealed on the bottom using a custom capping plate containing quick-turn fittings (McMasterCarr, Cat. No. 51525K372) after adding 2.5 mL of BHI growth medium, and then sealed on the top using push-in caps (McMasterCarr, Cat. No. 94075K56). After vortexing for 10 min on a rack vortexer, 60 µL aliquots were taken from each

filter cup and transferred to a 96-well PCR plate ( $T_0$  aliquots). The cups were resealed on the top and incubated for 9 hr at 37 °C and 230 rpm. One milliliter aliquots were taken from each filter cup at the endpoint ( $T_9$  aliquots), after vortexing the filter cup manifold on a platform vortexer for 10 min.

When the RV-PCR protocol was performed manually, all liquid handling was effectuated with serological and micro-pipettes. In the automated version of the protocol, a robotic platform (Perkin-Elmer, Janus workstation) performed all the liquid handling steps required to implement the RV-PCR method (mixing and transferring buffer from sample extracts to filtration cups for spore collection, as well as performing washes on the filters, adding growth medium to the filter cups for culturing and sampling cultures for PCR analysis), with the exception of the initial sample spiking and magnetic bead-based DNA clean up.

**Magnetic bead-based DNA clean up.** The 1 mL sample aliquots taken after 9 hr of incubation ( $T_9$  aliquots) were manually processed using the Promega Magnesil magnetic bead DNA clean up kit (Promega Co., Cat. No. MD1360, Technical Bulletin 312). Briefly, 1 mL of each sample was transferred from the filter cup into a 2 mL eppendorf tube, followed by addition of 600  $\mu$ L of bead mix and 360  $\mu$ L of lysis buffer. Sample, buffer and magnetic beads were mixed by pipetting and tubes were mounted on a tube rack interfaced with a magnet (Invitrogen, DynaMag™-2 magnet, Cat. No. 123-21D). Beads with attached DNA were attracted to the magnet and the supernatant was removed by pipetting. An additional lysis with 360  $\mu$ L of lysis buffer was conducted with mixing by pipetting and removal of the supernatant. Two washes with 360  $\mu$ L of salt solution were then performed, followed by mixing by pipetting and removal of the supernatant. Finally, two washes with 360  $\mu$ L of alcohol wash solution were performed with mixing and supernatant removal. Beads were allowed to air-dry for 2 min, followed by transfer

of the tube rack from the magnetic support to a hot plate and heating for 10 min at 80 °C. DNA elution/concentration was then performed by adding 200 µL of elution buffer while sample tubes remained on the hot plate. The sample with buffer was mixed and transferred to the magnetic support, and the supernatant with eluted DNA was recovered (typically 80 µL). A ten-fold dilution of the eluted sample in PCR-grade water was systematically performed using PCR-grade water and a 96-well Bioblock (E&K Scientific, Cat. No. 662000) prior to running PCR in order to counter inhibition from the environmental background (all samples were processed with a 1:10 dilution for consistency).

**Extracted DNA controls.** DNA controls were generated for the *B. anthracis* Ames strain. DNA was extracted from cultured cells using a complete DNA and RNA purification kit (Epicentre Biotechnologies, MasterPure™, Cat. No. MC85200) and DNA concentration was measured with a Qubit™ fluorometer (Invitrogen, Cat. No. Q32857) using the PicoGreen™ assay (Invitrogen, Cat. No. Q32851). Standard concentrations ranging from 1 ng/µL to 1 fg/µL were prepared in PCR-grade water. Seven 10-fold dilutions, ranging from 5 ng per 25-µL PCR reaction to 5 fg per 25-µL PCR reaction, were run with each set of PCR plates.

**PCR.** Five micro-liter sample aliquots were transferred to a 96-well PCR plate with 20 µL of PCR mix. PCR mix was prepared for each of the 3 primer-probe sets using 12.5 µL of TaqMan™ 2X Universal Master Mix (ABI Cat. No. 4305719) and 1 µL of 2 µM probe solution. For the chromosome and pXO1 assays, 1 µL of a 25 µM solution was used for each primer. For the pXO2 assays, 0.3 µL of 25 µM solution was used for each primer. The volume of PCR mix was completed to 20 µL using PCR-grade water. After mixing and centrifugation, PCR was run using the ABI 7500 Fast platform (Applied Biosystems, United States) in fast mode. Thermal

cycling parameters were as follows: 2 min at 50 °C for Uracil-N-Glycosilase incubation, 10 min at 95 °C for AmpliTag™ gold activation, followed by 45 amplification cycles (5 s at 95 °C for denaturation and 20 s at 60 °C for annealing/extension). Each sample was analyzed against each of the 3 primer/probe sets. A minimum of 4 sample replicates were analyzed for each set of experimental conditions. The ROX reference dye contained in the ABI universal mastermix was used to normalize the fluorescent reporter signal.

**RV-PCR Data analysis.** Control experiments performed by aliquotting 1 mL from each filter cup immediately after addition of BHI growth medium (T<sub>0</sub>) followed by vortexing and processing aliquots with the magnetic bead-based DNA clean up method described above showed no detectable PCR signal for any of the 3 *B. anthracis* assays for spiking levels up to 10<sup>5</sup> CFU/samples. These results confirmed that the DNA clean up procedure does not lyse *B. anthracis* spores and therefore, that no DNA is released for PCR at T<sub>0</sub>. Similar controls were performed on samples inoculated with 10<sup>6</sup> dead *B. anthracis* spores, again showing an absence of any measurable Ct values at T<sub>0</sub>. All results from this study were therefore presented as Ct values after 9 hr of incubation (T<sub>9</sub>). Each Ct represents the average of 4 replicate samples. Standard deviations were reported for each Ct value.

## RESULTS AND DISCUSSION

**PCR assays.** Potential assays were ranked and down-selected using *in silico* analysis for signature specificity against all available sequences in Genbank, virulence gene association, availability of prior assay screening data and amplicon characteristics. The output of this analysis was a computational prediction of virulent *B. anthracis* strain detection for 44 candidate assays ranked for predicted selectivity, amplicon size, and gene target. Ten assays (3 for the

chromosome, 4 for the pXO1 plasmid and 3 for the pXO2 plasmid) were down-selected based on the *in-silico* analysis and then optimized for real-time PCR. Three assays (1 for the chromosome targeting a hypothetical protein, 1 for the pXO1 plasmid targeting a hypothetical protein and 1 for the pXO2 plasmid targeting the capsule biosynthesis protein cap b) were ultimately selected for RV-PCR based on sensitivity, selectivity and robustness in the presence of growth medium and cell debris.

Assays sensitivity with extracted *B. anthracis* Ames DNA was shown to be below 10 genome copies for the 3 selected assays (Table 1). Assay specificity was tested using a panel of 13 *B. anthracis* strains (Turkey 32, A0149, A0248, V770-NP-1R, Ba1015, SK-102, Ba1035, K3, PAK-1, RA3, Vollum 1B, Sterne, Ames) and 15 *B. anthracis* near neighbors (*B. cereus* [S2-8, 3A, E33L, D17, FM1, 03BB102, 03BB108], *Bacillus thuringiensis* [HD1011, 97-27, HD682, HD571], *B. thuringiensis* *Israelensis*, *B. thuringiensis* *Kurstaki*, *B. thuringiensis* *Morrisoni*, *Bacillus Al Hakam*). The 3 assays detected the 13 *B. anthracis* strains tested. No cross-reactivity was observed, with the exception of the 2 plasmid assays, which detected *Bacillus cereus* 03BB102 and 03BB108. Finally, the robustness of the 3 assays in the presence of growth media and cell debris was tested by diluting extracted *B. anthracis* Ames DNA in a lysed culture of *B. atrophaeus* ( $10^9$  CFU/mL). The variation in Ct value induced by the presence of growth medium and cell debris compared to PCR-grade water was typically below 1.0, confirming that the assay performance was adequate for use in the RV-PCR method.

**Detection of live *B. anthracis* Ames spores on clean samples.** Manual and automated RV-PCR experiments were performed on clean wipe, air filter, and water samples spiked with live *B. anthracis* Ames at levels of 10 and 100 CFU/sample. Fig. 2 summarizes the Ct values obtained for clean samples spiked at the 10 live *B. anthracis* Ames spore level with each of the 3 assays.

Ct values at T<sub>9</sub> were below 35.0 for all samples and all assays, indicating a limit of detection at or below the 10 spore level on clean samples. It should be noted that at this low spiking level (10 CFU/sample, which becomes 10 spores/20 mL of buffer in the sample tube), plating of the samples directly from the sample tube or from the filter cup after addition of growth medium did not lead to any detectable colonies after 24 hr of incubation on BHI agar. This result exemplifies the advantage of the RV-PCR method over the plating method (where the entire sample volume is not typically plated) for the detection of low levels of live *B. anthracis*. Culturing of sample aliquots drawn from filter cups after 9 hr of incubation on BHI agar plates showed that samples went from being undetectable to a level of 10<sup>7</sup> CFU/mL in 9 hr. Such growth corresponds to a doubling time in the order of 30 min, assuming a germination time in the 30-60 min range, which suggests optimal growth conditions for *B. anthracis* in this high throughput format (filter cups) and confirms that the Ct values recorded at T<sub>9</sub> originate from viable spores.

The plasmid assays consistently generated slightly lower Ct values than the chromosome assay, which may be attributed to the higher number of plasmid copies relative to the chromosome (13). Results provided by the manual and automated methods were not statistically different, which suggests that automation may be used to reduce labor and risk of personnel exposure without compromising the limit of detection of the RV-PCR method.

**Detection of live *B. anthracis* Ames spores in the presence of dirt and/or debris.** Manual and automated RV-PCR experiments were performed on dirty wipe, air filter, and water samples spiked with live *B. anthracis* Ames at levels of 10 and 100 CFU/sample. As described in the Materials and Methods section, dirty wipes were prepared by adding 250 mg of Ultra Fine Arizona Test Dust, dirty air filters came from air sampling units (operated at 85-100 lpm for 24 hr), and dirty water was prepared by spiking Milli-Q<sup>TM</sup>-filtered water with 10 mg/L of humic

acid and 10 mg/L of ferrous sulfate, which are known PCR inhibitors (14-16). Fig. 3 summarizes the Ct values obtained for dirty samples spiked at the 10 live *B. anthracis* Ames spore level with each of the 3 assays. Although higher Ct values were obtained on dirty samples relative to clean samples, all spiked samples were detected, with T<sub>9</sub> Ct values below 36.0 for all assays, indicating that the limit of detection obtained for clean samples was maintained in the presence of dirt and/or debris. It should be noted that all samples are diluted by a factor 10 in PCR-grade water after the magnetic bead-based DNA clean up step, as described in the Materials and Methods section. This additional step was added in the method in order to counter PCR inhibition from environmental compounds, such as humic acid, ferrous sulfate and metal oxides present in water and dust/soil (typical Ct values at the 10 spore level without dilution are in the 37-45 range on dirty samples while typical Ct values after 1:10 dilution are in the 30-36 range on the same samples). The results provided by the manual and automated methods were not statistically different, as previously observed on clean samples, which suggests that the use of high-throughput automation is appropriate for environmental samples. In addition, filtration times for dust-containing samples remained rapid since the filter cups have a large filtration area (4.7 cm<sup>2</sup>).

**Detection of live *B. anthracis* Ames spores in the presence of a high background of dead *B. anthracis* Ames spores.** Overcoming the challenge posed by high levels of dead *B. anthracis* spores is critical for remediation activities involving post-decontamination clearance. In order to restore sites after decontamination, very low levels of live spores must be detected in a high background of spores killed by decontamination activities. Manual and automated RV-PCR experiments were performed on clean wipe, air filter, and water samples spiked with live *B. anthracis* Ames at levels of 10 and 100 CFU/samples in a background of 10<sup>6</sup> dead *B. anthracis* Ames spores/sample killed by autoclaving. Fig. 4 summarizes the T<sub>9</sub> Ct values obtained at the 10

live *B. anthracis* Ames spore level with each of the 3 assays. For all sample types, Ct values were below 35.0 for all assays, indicating that a level of 10 live *B. anthracis* Ames spores per sample was detected in a background of  $10^6$  dead spores. Interferences of the decontamination method (fumigants or liquid disinfectants) with the RV-PCR method were not tested in this study, however, earlier studies with *B. anthracis* surrogates showed no impacts of residual fumigant (8). Typical effects of decontamination including delayed germination and growth and PCR inhibition can usually be overcome by increased incubation time and sample dilution (8). The RV-PCR protocol includes washes of the spores in the filter cups in order to remove any residual disinfectant.

**Detection of live *B. anthracis* Ames spores in the presence of a high background of live non-target organisms.** Manual and automated RV-PCR experiments were performed on clean wipe, air filter, and water samples spiked with live *B. anthracis* Ames at levels of 10 and 100 CFU/sample in a combined background of  $10^3$  live *B. atrophaeus* and  $10^6$  *P. aeruginosa* CFU/sample. Fig. 5 summarizes the  $T_9$  Ct values obtained at the 10 live *B. anthracis* Ames spore level for each of the 3 assays. For all sample types, Ct values were below 35.0, indicating that the presence of a high background of live organisms did not negatively impact the limit of detection of the method. The ability to detect a live target organism in a complex environmental background is provided by the selectivity of the PCR assays and constitutes a critical advantage of the RV-PCR method over the standard plating method, in which agar plates may be overwhelmed by the growth of live non-target organisms. The sample extraction buffer contains 30% ethanol in order to lyse vegetative cells (while spores were shown to remain intact and viable in this buffer) and prevent a competition for growth medium in the filter cups.

**Relationship between incubation time and limit of detection.** Three additional RV-PCR experiments were performed in order to further explore the relationship between the limit of detection of the RV-PCR method and the incubation time. Three spore levels ( $10^3$ ,  $10^2$  and  $10^1$  CFU/sample) were spiked on clean wipes and samples were processed with the RV-PCR method using incubation times ranging from 8 to 6 hr. Seven replicate samples were analyzed for each set of experimental conditions. Results from these experiments are summarized in Fig. 6. As expected, the shorter the incubation time, the lower the DNA concentration and the higher the Ct value. The only Ct values above 35.0 were: the chromosome assay for the T<sub>7</sub> experiment at the 10 spore level, the chromosome and pXO1 assays for the for the T<sub>6</sub> experiment at the 10 spore level, and the chromosome assay for the T<sub>6</sub> experiment at the 100 spore level. These results suggest that depending on the criteria used to determine whether a sample is positive for viable *B. anthracis* spores (number of assays and Ct threshold) and the desired limit of detection, the incubation time could potentially be reduced to 7 or 6 hr when processing relatively clean samples, reducing the total turnaround time of the method to 12-13 hr.

**Conclusions.** A verified RV-PCR method was presented for detection of live, virulent *B. anthracis* spores in wipe, air filter, and water samples. The method endpoint was shortened from its initial overnight incubation (14 hr) to 9 hr by performing a magnetic bead-based DNA clean up procedure before PCR analysis. Using this method, the total processing time from start to finish for 24 samples was reduced to 15 hr (2-3 hr of processing time should be added for each additional set of 24 samples when processing samples in series with a single robot), which is significantly shorter than the standard plating method, which requires up to several days to obtain confirmed results. In addition, we report the extension of the RV-PCR method to virulent *B. anthracis* using 3 specific assays. Manual and automated versions of the method showed

limits of detection at the 10 spore level with and without debris for all three sample types. Live *B. anthracis* Ames spores were consistently detected at the 10 spore level for both manual and automated methods in dead *B. anthracis* spore backgrounds of  $10^6$  spores/samples and live, combined non-target backgrounds of  $10^3$  *B. atrophaeus* and  $10^6$  *P. aeruginosa* CFU per sample.

In addition to the shorter turnaround time and lower detection limit, the RV-PCR method also presents operational advantages over the plating method including a smaller footprint (one table top incubator is sufficient to incubate up to 96 samples), a reduction in labor per sample, and a higher throughput (~150 samples may be analyzed in 24 hr when proceeding with serial analysis with a single robot and 2 personnel).

Based on the advantages described above, the RV-PCR method may constitute a new capability to ensure public safety, but other potential applications may also lie in surveillance, public health, and food safety.

## ACKNOWLEDGEMENTS

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## REFERENCES

1. **Raber, E., A. Jin, K. Noonan, R. McGuire, and R. D. Kirvel.** 2001. Decontamination issues for chemical and biological warfare agents: how clean is clean enough? *Int. J. Environ. Health Res.* **11**:128–148.
2. **Raber, E., T. M. Carlsen, K. J. Folks, R. D. Kirvel, J. I. Daniels, and K. T. Bogen.** 2004. How clean is clean enough? Recent developments in response to threats posed by chemical and biological warfare agents. *Int. J. Environ. Health Res.* **14**:31–41.
3. **Raber, E., J. M. Hirabayashi, S. P. Mancieri, A. L. Jin, K. J. Folks, T. M. Carlsen, and P. Estacio.** 2002. Chemical and biological agent incident response and decision process for civilian and public sector facilities. *Risk Anal.* **22**:195–202.
4. **Fitch, J. P., E. Raber, and D.R. Imbro.** 2003. Technology challenges in responding to biological or chemical attacks in the civilian sector. *Science* **302**:1350–1354.
5. **Hodges, L. R., L.J. Rose, A. Peterson, J. Noble–Wang, and M. J. Arduino.** 2006. Evaluation of a macrofoam swab protocol for the recovery of *Bacillus anthracis* spores from a steel surface. *Appl. Environ. Microbiol.* **72**:4429–4430.
6. **Hodges, L. R., L. J. Rose, H. O’Connell, and M. J. Arduino.** 2010. National Validation Study of a Swab Protocol for the Recovery of *Bacillus anthracis* Spores from Surfaces. *J. Microbiol. Methods* **81**:141–146.
7. **Buttner, M. P., P. Cruz, L. D. Stetzenbach, A. K. Klima–Comba, V. L. Stevens, T. D. Cronin.** 2004. Determination of the efficacy of two building decontamination strategies by surface sampling with culture and quantitative PCR analysis. *Appl. Environ. Microbiol.* **70**:4740–4747.

8. **Kane, S. R., S. E. Létant, G. A. Murphy, T. M. Alfaro, P. W. Krauter, R. Mahnke, and E. Raber.** 2009. Rapid, high-throughput, culture-based PCR methods to analyze samples for viable spores of *Bacillus anthracis* and its surrogates. J. Microbiol. Methods **76**:278-284.
9. **Létant, S. E., S. R. Kane, G. A. Murphy, T. M. Alfaro, L. R. Hodges, L. J. Rose, and E. Raber.** 2010. Most Probable Number Rapid Viability PCR Method to Detect Viable Spores of *Bacillus anthracis* in Swab Samples. J. Microbiol. Methods **81**:200-202.
10. **Materials Safety Data Sheet.** Arizona Test Dust, 2006. Powder Technology Inc., 14331 Ewing Avenue S., Burnsville, MN 55306.  
[http://www.powdertechinc.com/pages/english/PTI\\_MSDS.html](http://www.powdertechinc.com/pages/english/PTI_MSDS.html)
11. **Hodges L. R., L. Rose, H. O'Connell and M. J. Arduino.** 2009. National validation study for a swab protocol for the recovery of *Bacillus anthracis* spores from surfaces, ASM Biodefense conference, poster.
12. Unpublished results.
13. **Rasko, D. A., M. J. Rosovitz, O. A. Økstad, D. E. Fouts, L. Jiang, R. Z. Cer, A.-B. Kolstø, S. R. Gill, and J. Ravel.** 2007. Complete sequence analysis of novel plasmids from emetic and periodontal *Bacillus cereus* isolates reveals a common evolutionary history among the *B. cereus*-group plasmids, including *Bacillus anthracis* pXO1. J. Bacteriol. **189**:52-64.
14. **Kreader, C. A.** 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 Gene 32 protein. Appl. Environ. Microbiol. **62**:1102-1106.

- 454 15. **Tsai, Y.-L., and B. Olson.** 1992. Rapid method for separation of bacterial DNA from  
455 humic substances in sediments for polymerase chain reaction. *Appl. Environ. Microbiol.*  
456 **58**:2292-2295.
- 457 16. **Teng, F, G.** Yuntao, and Z. Wanpeng. 2008. A simple and effective method to overcome  
458 the inhibition of Fe to PCR. *J. Microbiol. Methods* **75**:362-364.
- 459

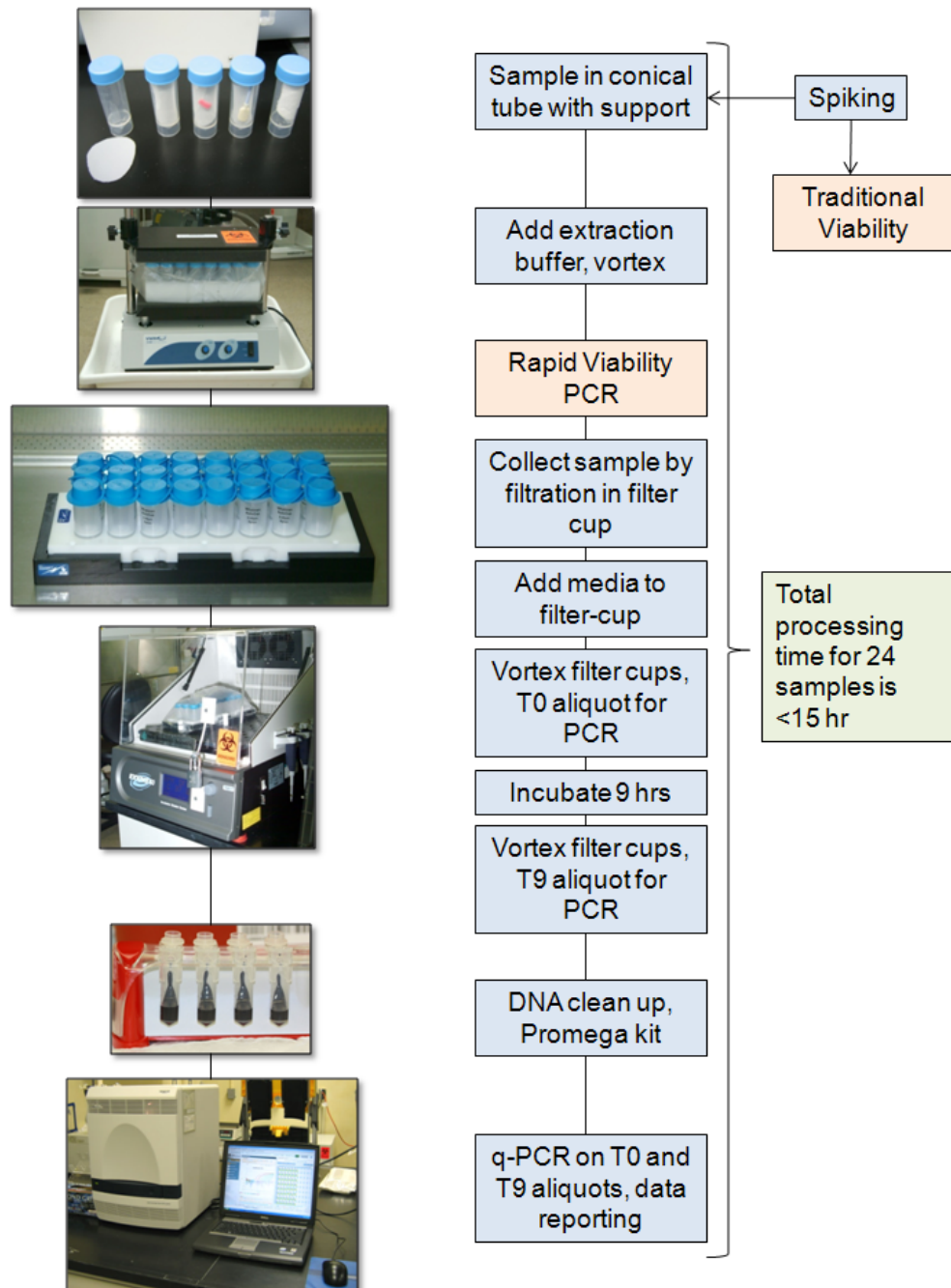


FIG. 1. Summary of the RV-PCR protocol steps and pictures of the equipment used to process samples.

TABLE 1. Cycle threshold values and corresponding standard deviations for down-selected PCR assays, tested with extracted *Bacillus anthracis* Ames DNA.<sup>a</sup>

DNA (pg) <sup>b</sup>	Genome copy no. <sup>c</sup>	Cycle threshold		
		PCR assay		
		Chromosome	pXO1	pXO2
5000	829000	19.4 ± 0.2	20.7 ± 0.2	18.5 ± 0.1
500	82900	22.6 ± 0.1	23.1 ± 0.1	21.4 ± 0.1
50	8290	26.5 ± 0.1	26.4 ± 0.1	24.7 ± 0.1
5	829	30.6 ± 0.1	29.9 ± 0.2	28.4 ± 0.1
0.5	82.9	34.6 ± 0.1	33.4 ± 0.1	31.9 ± 0.1
0.05	8.29	38.5 ± 0.4	37.2 ± 0.6	35.4 ± 0.3

<sup>a</sup>Ct values are averages of 3 replicate reactions.

<sup>b</sup>DNA was quantified by fluorimetry using the PicoGreen™ assay.

<sup>c</sup>Genome copies are based on estimated genome size of 5.5 Mb.

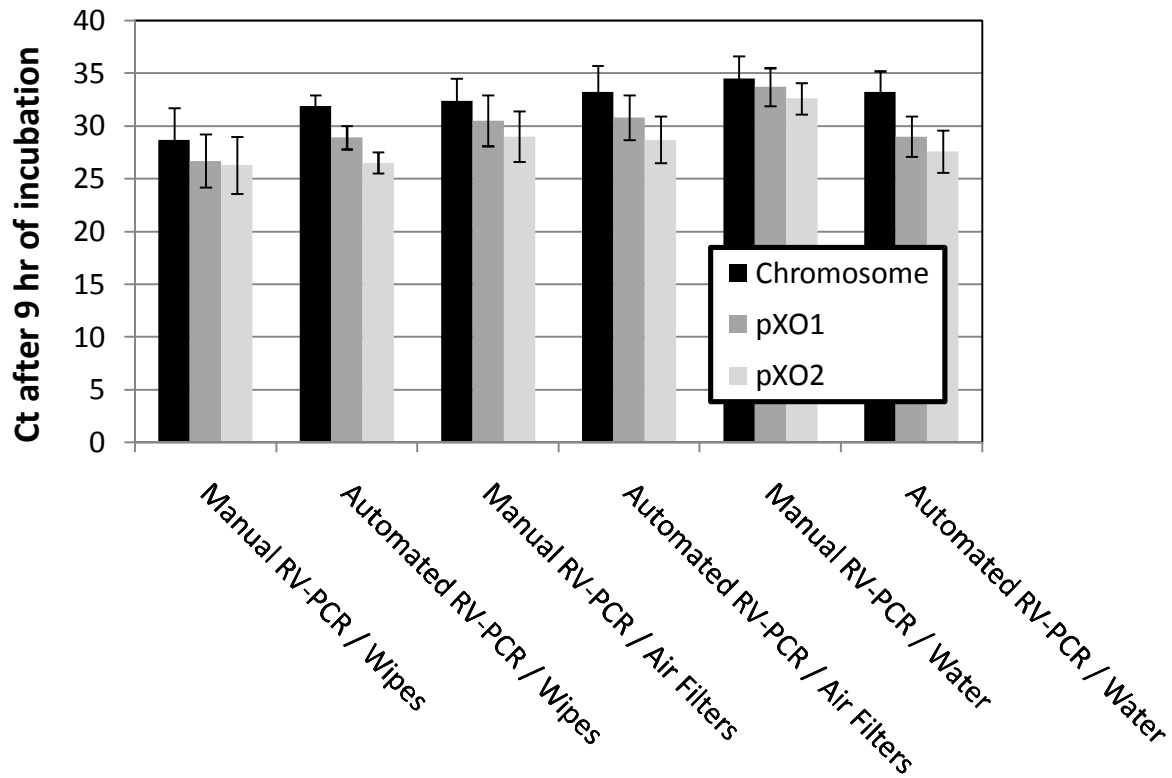
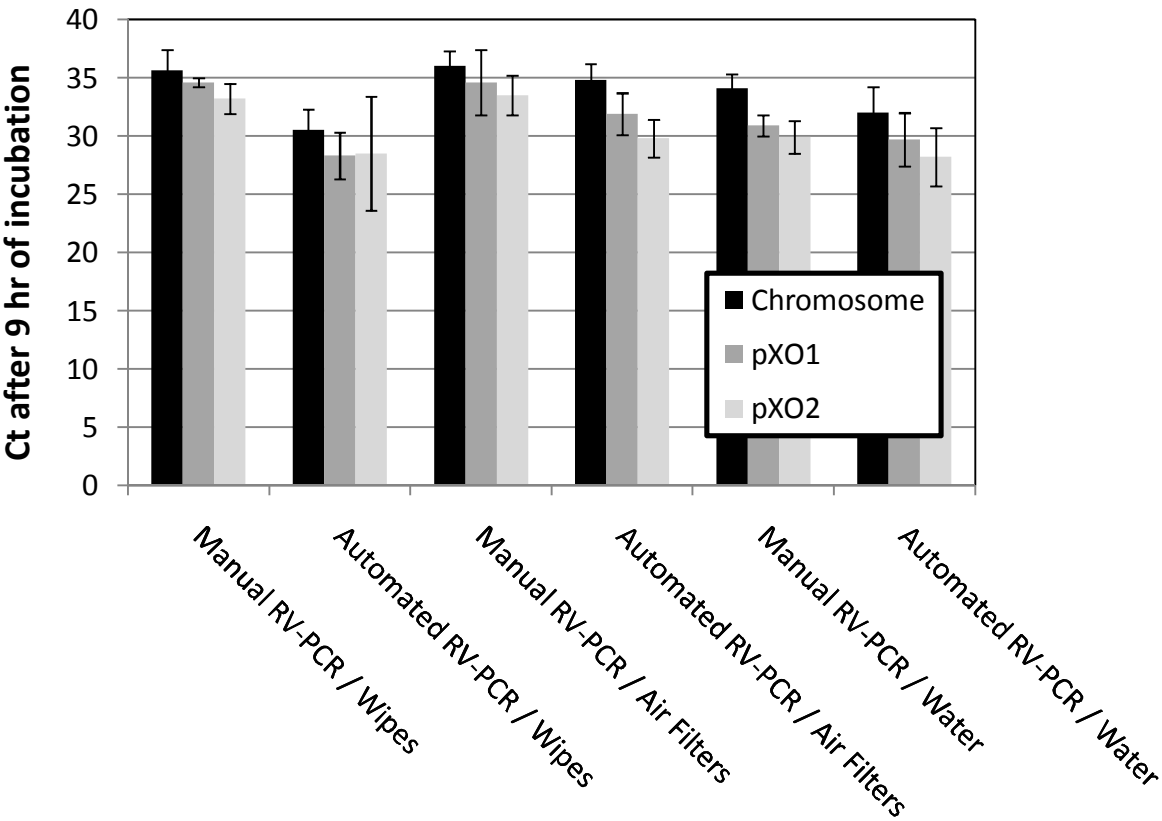


FIG. 2. Manual and automated RV-PCR results obtained on clean wipe, air filter and water samples spiked with live *Bacillus anthracis* Ames at a level of 10 CFU/samples (samples processed with the manual protocol were spiked with  $31 \pm 2$  CFU/sample and samples processed with the automated protocol were spiked with  $26 \pm 1$  CFU/sample, as measured by plating). Each Ct value is the average of 4 replicate samples. Error bars represent one standard deviation above and below the average Ct value.



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FIG. 3. Manual and automated RV-PCR on dirty wipe, air filter and water samples spiked with live *Bacillus anthracis* Ames at a level of 10 CFU/samples (samples processed with the manual protocol were spiked with  $49 \pm 3$  CFU/sample and samples processed with the automated protocol were spiked with  $40 \pm 2$  CFU/sample, as measured by plating). Dirty wipes were prepared by adding 250 mg of Ultra Fine Arizona Test Dust, dirty air filters came from a portable air sampling unit that operated for 24 hr at approx. 200 lpm and dirty water was prepared by spiking sterile, filtered water with 10 mg/L of humic acid and 10 mg/L of ferrous sulfate. Each Ct value is the average of 4 replicate samples. Error bars represent one standard deviation above and below the average Ct value.

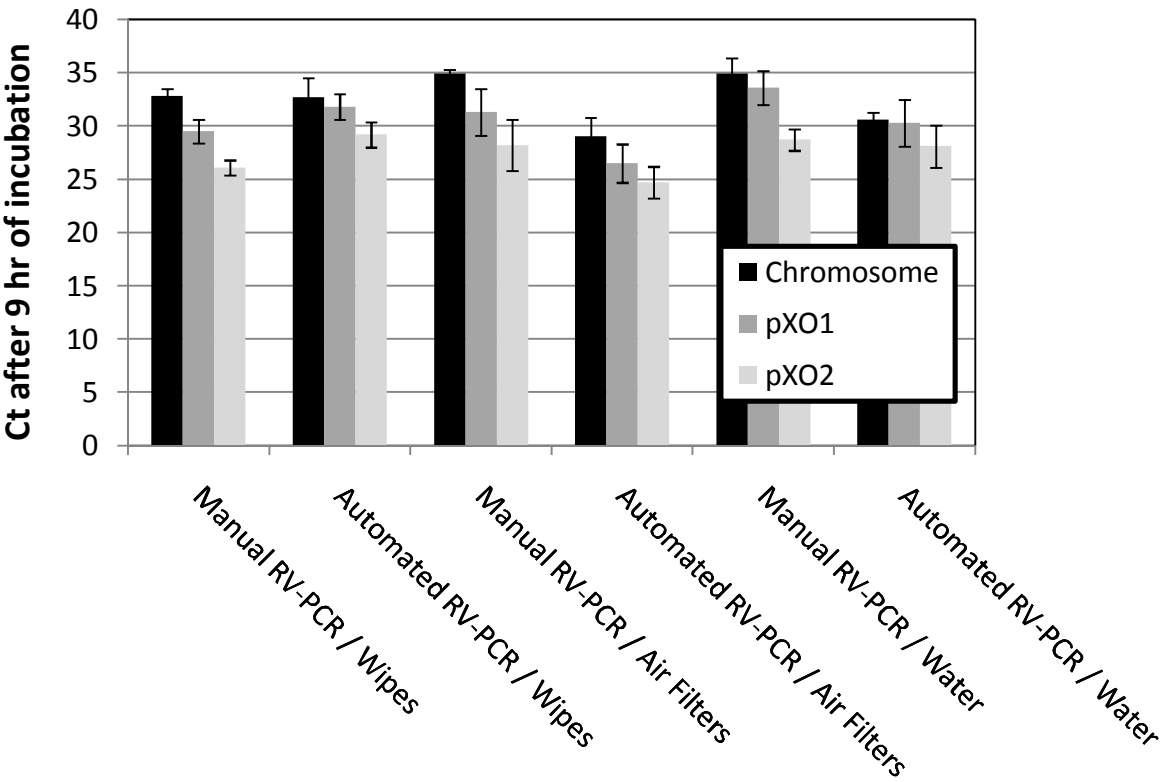
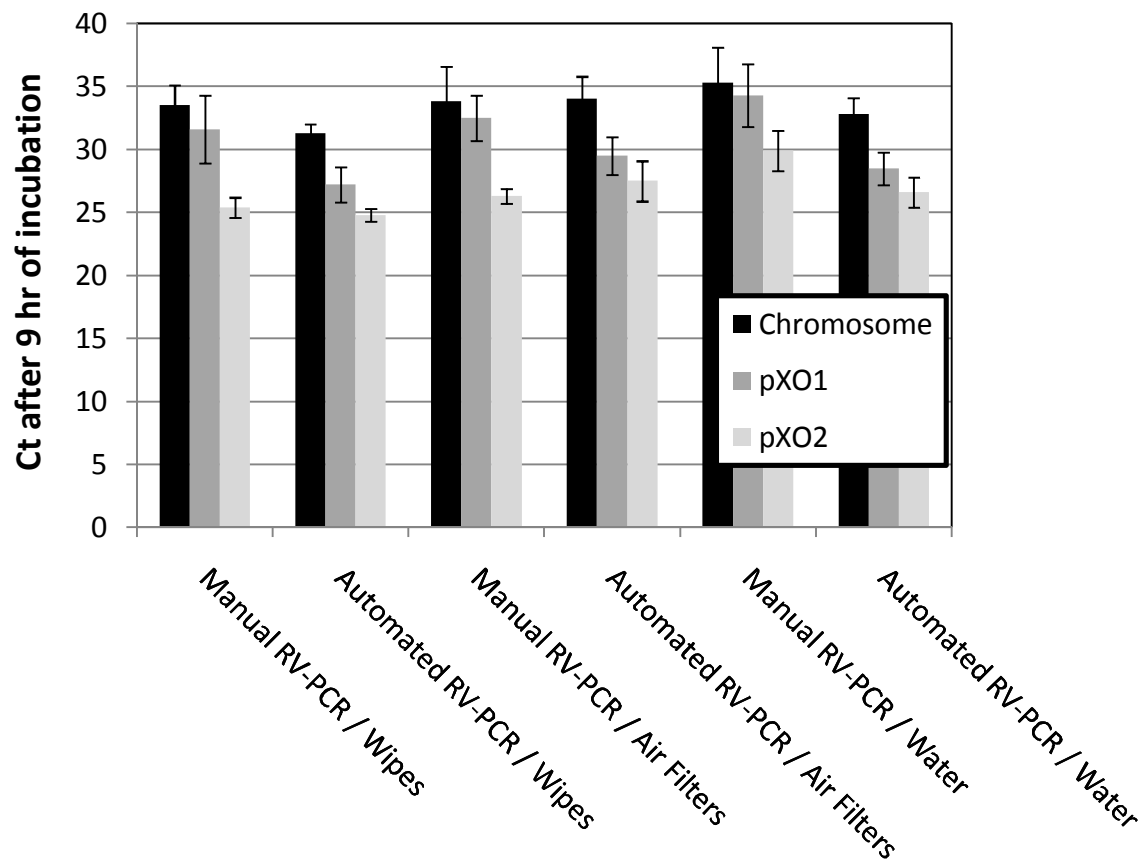


FIG. 4. Manual and automated RV-PCR results obtained on clean wipe, air filter and

water samples spiked with live *Bacillus anthracis* Ames at a level of 10 CFU/samples (samples processed with the manual protocol were spiked with  $14 \pm 1$  CFU/sample and samples processed with the automated protocol were spiked with  $38 \pm 2$  CFU/sample, as measured by plating) in a background of  $10^6$  dead *Bacillus anthracis* Ames spores/sample. Each Ct value is the average of 4 replicate samples. Error bars represent one standard deviation above and below the average Ct value.

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FIG. 5. Manual and automated RV-PCR results obtained on clean wipe, air filter and water

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samples spiked with live *Bacillus anthracis* Ames at a level of 10 CFU/samples (samples

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processed with the manual protocol were spiked with  $33 \pm 2$  CFU/sample and samples processed

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with the automated protocol were spiked with  $21 \pm 1$  CFU/sample, as measured by plating) in a

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combined background of  $10^3$  live *B. atrophaeus* and  $10^6$  *P. aeruginosa* CFU/sample. Each Ct

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value is the average of 4 replicate samples. Error bars represent one standard deviation above and

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below the average Ct value.

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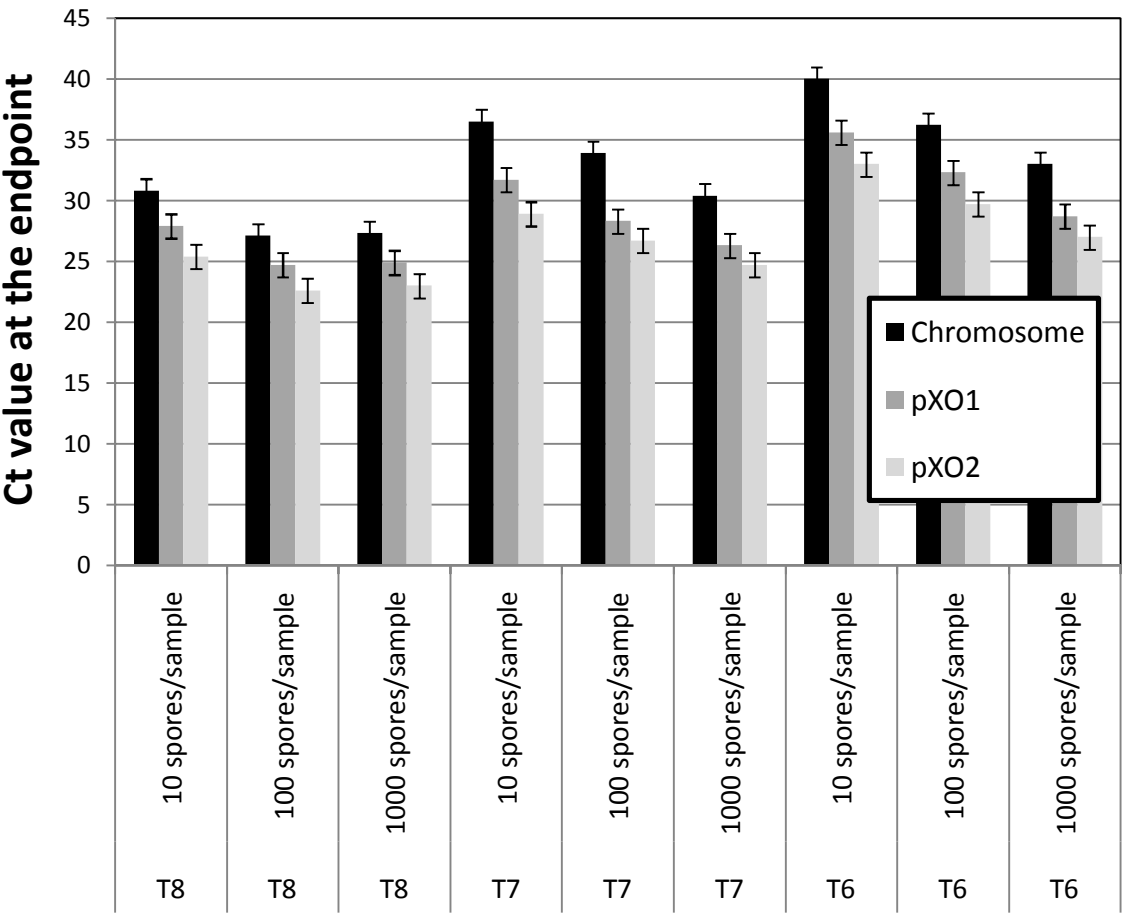


FIG. 6. Manual RV-PCR results obtained on clean wipe samples spiked with 3 levels of live *Bacillus anthracis* Ames ( $54 \pm 9$  CFU/sample,  $813 \pm 43$  CFU/sample, and  $81300 \pm 430$  CFU/samples). The incubation time was varied from 8 to 7 and then 6 hr (noted T8, T7 and T6 respectively). Each Ct value is the average of 7 replicate samples. Error bars represent one standard deviation above and below the average Ct value.